

L-Selectin and β_2 -Integrin Expression on Circulating Bovine Polymorphonuclear Leukocytes During Endotoxin Mastitis

A. Diez-Fraile, E. Meyer, L. Duchateau, and C. Burvenich

Department of Physiology, Biochemistry and Biometrics, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

ABSTRACT

The aim of this in vivo study was to examine the effect of intramammarily administered endotoxin (lipopolysaccharide, LPS) on the expression of L-selectin (CD62L) and the β_2 -integrin subunits CD11b and CD18 on circulating bovine PMN. Six early lactating cows were infused with *Escherichia coli* LPS. The adhesion molecules under study were stained at the cell surface and analyzed flow cytometrically. In addition, some of the clinical parameters associated with adhesion molecule mobilization such as fever, blood cortisol levels, somatic cell count (SCC), and total and differential blood leukocyte count were measured. In analogy with observations during clinical coliform mastitis, a progressive decrease of CD62L expression levels was observed early after LPS infusion, concomitantly with a continuous rise of CD11b and CD18 density. However, no correlation was found between the kinetics of CD11b and CD18 density. The initial changes in adhesion molecule expression paralleled the decrease in blood PMN numbers, together with the increase in rectal temperature, cortisol levels, SCC, and number of circulating immature PMN. In conclusion, intramammarily administered LPS seems to play an important role in modulating adhesion receptor expression on circulating bovine PMN. Interestingly, in contrast to coliform mastitis, the net CD18 variation is not principally influenced by CD11b upregulation during endotoxin administration. The knowledge of adhesion molecule kinetics in relation to the different parameters evaluated in the present study contributes to an improved understanding of the inflammatory reaction.

(Key words: endotoxin mastitis, adhesion molecule, polymorphonuclear leukocyte, bovine)

Abbreviation key: FITC = fluorescein isothiocyanate, IL = interleukin, LPS = lipopolysaccharide or endo-

toxin, MFI = mean fluorescence intensity, PMN = polymorphonuclear leukocyte, TNF = tumor necrosis factor.

INTRODUCTION

Escherichia coli is a major pathogen that instigates severe clinical mastitis during early lactation. It has been suggested that the endotoxin (lipopolysaccharide, LPS) released during the exponential growth and subsequent death of the bacteria in the mammary gland is the main determinant of the degree of the inflammatory reaction. Indeed, intramammary infusion with LPS leads to the development of acute mastitis, which is clinically similar to the naturally occurring infection. However, LPS mastitis, as compared to coliform mastitis, is a milder disease from which all infused animals completely recover in the absence of a curative treatment (Lohuis et al., 1988; Dosogne, 1998; Hoebe et al., 2000). As a matter of fact, a different pattern of polymorphonuclear leukocyte (PMN) invasion and udder lesions can be observed by electron microscopy in the two different types of mastitis (Hill, 1994). This particular suggests that the PMN migratory process during *E. coli* and endotoxin mastitis is likely to be different. It has been previously proposed, however, that the activation and migration of PMN to the inflammatory focus is mainly due to the release of secondarily mediators (Lohuis et al., 1988; Dosogne, 1998; Hoebe et al., 2000), thereby establishing a link between the two types of mastitis.

Bovine PMN express two important classes of adhesion molecules, i.e., L-selectin (CD62L) and β_2 -integrins (CD11/CD18), which mediate the migration of activated circulating PMN through the blood-milk barrier. After PMN activation, CD62L is rapidly shed from the cell membrane, a necessary process for subsequent firm adhesion to the vascular endothelium. Activation of the bovine PMN adhesion receptor CD11/CD18 is essential for PMN accumulation at the inflammatory site (for a review, see Diez-Fraile et al., 2002). Of the various β_2 -integrin subunits, we studied CD11b and CD18, which are critical for diapedesis through bovine mammary endothelial and epithelial cells (Smits et al., 2000). After intramammary infection with *E. coli*, the CD62L

Received October 3, 2002.

Accepted January 22, 2003.

Corresponding author: C. Burvenich; e-mail: Christian.burvenich@rug.ac.be.

expression level diminishes (Monfardini et al., 1999; Diez-Fraile et al., 2000b), and CD11b and CD18 density increases on bovine blood PMN (Dosogne et al., 1997; Roets et al., 1999). At present, only a few studies have partially evaluated the role of intramammary infused LPS on CD62L and CD11/CD18 expression (Paape et al., 1996; Yagi et al., 2002).

Detailed insight into the mechanisms of adhesion molecule modulation on PMN is essential for a global understanding of the pathogenesis of mastitis. The present in vivo model aims to closely examine the role of endotoxin on adhesion molecule kinetics. The significance of our findings in relation to several parameters involved in the modulation of CD62L, CD11b, and CD18 adhesion molecules is discussed.

MATERIALS AND METHODS

Animals

Experiments were performed on six clinically healthy, high-yielding cows of the East-Flemish Red Pied breed. All cows were in their first lactation and between 2 and 6 wk after parturition. Only cows with milk SCC lower than 100,000 cells/ml in each individual quarter and negative for pathogens based on two consecutive bacteriological milk analyses were accepted for the study. Cows were transferred to individual stalls 1 wk before the start of the experiment. They were fed a daily ration of 8 kg of concentrate and had free access to water and hay. Animals were milked twice daily at 7 a.m. and 4 p.m. with a four-quarter milking machine.

Endotoxin Infusion

Ten milligrams of LPS derived from *E. coli*, strain O111:B4 (Sigma Chemical Co., St. Louis, MO), was diluted in 100 ml of pyrogen-free saline solution and divided into 5-ml aliquots (500 µg of LPS/5 ml). Air was removed from the bottles by a flow of sterile N₂ gas and these LPS solutions were stored at -20°C until use. Before LPS injection, the teat ends were disinfected with ethanol (70%) mixed with chlorohexidine. LPS aliquots were thawed immediately before use, and 15 ml of pyrogen-free saline solution was added. On d 0, 1 h after the morning milking, six cows were injected through the streak canal in the left front and rear quarters with 500 µg of LPS diluted in 20 ml of saline solution per quarter using a sterile teat cannula. After infusion, each quarter was massaged to distribute the endotoxin solution throughout the gland. The study was approved by the Ethical Committee of the Faculty of Veterinary Medicine at the Ghent University.

Blood and Milk Sampling

Peripheral blood samples were taken by venipuncture from the external jugular vein into evacuated tubes (BD Vacutainer, Plymouth, UK) containing heparin as anticoagulant. Blood samples were collected on d -3, -2, -1, 0, 1, 2, 3, 6, and 9 with respect to the day of infusion. On d 0, additional samples were gathered every hour from 1 until 10 h, and at 12 and 14 h after intramammary challenge. At the same time, rectal temperature and other clinical signs were monitored. Quarter milk samples were aseptically obtained from each animal on d -1, 0, 1, 2, 3, 6, and 9. Additional samples were collected on d 0, each hour from 1 until 10 h, and at 12 and 14 h after infusion.

Cortisol Determination

Plasma cortisol concentrations were determined by radioimmunoassay (Sulón et al., 1978). Briefly, 100 µl of plasma was extracted with 3 ml of dichloromethane. One milliliter of the organic phase was subjected to evaporation and the radioimmunological procedure was performed on the dried residue. A standard curve (ranging from 10 pg to 1 ng of cortisol/tube) was prepared in parallel. After incubation overnight at 4°C, the bound and free fractions of tracer were separated by the addition of a sheep anti-rabbit IgG serum coupled to activated cellulose (DASP system: double antibody solid phase). After centrifugation and discarding of the supernatant, the radioactivity of the pellets was measured in a multigamma scintillation counter (Wallac 1261, Breda, The Netherlands).

SCC, Blood Leukocyte Count, and Blood PMN Maturity

The SCC was determined for each individual quarter by means of the fluoro-optoelectronic cell counting principle (Fossomatic 360; Foss Electronic, Eden Prairie, MN). Total leukocyte count was determined electronically (Coulter Counter ZF; Coulter Electronics Ltd., Luton, UK).

Smears were prepared from whole blood and stained with Eosin-Giemsa (Hemacolor, E. Merck, Darmstadt, Germany). Differential microscopic counts were determined by counting a total of 100 cells. Proportions of mature, band, and immature (i.e., myelocytes and metamyelocytes) PMN were evaluated.

Monoclonal Antibodies and Immunofluorescence Labeling

Blood aliquots of 100 µl were incubated in polystyrene round-bottom tubes (Becton Dickinson, San José, CA)

for 30 min at 37°C with 50 μ l of anti-bovine CD62L (clone 11G10; Wang et al., 1997), anti-bovine CD11b (clone CC126; ProBio, Margate Kent, UK), or anti-bovine CD18 mAb (clone MF14B4; Facultés Universitaires Notre Dame de la Paix, Immunology Unit, Namur, Belgium; Letteson and Delcommenne, 1993) at saturating concentrations. Control samples were incubated with 50 μ l RPMI 1640 (Gibco Brl., Scotland, UK) containing 1% bovine albumin fraction V (Merck KG&A, Darmstadt, Germany) and 0.2% sodium azide. After incubation, red blood cells were lysed for 6 min at room temperature with 300 μ l of 21.47 mM Trizma Base (Sigma-Aldrich) and 138.34 mM NH_4Cl (Merck). After centrifugation ($200 \times g$ for 10 min at 4°C), leukocytes were washed twice in 300 μ l of control solution. A second incubation was performed in the dark for 30 min at 4°C with 50 μ l of a goat anti-mouse IgG fluorescein isothiocyanate (FITC)-labeled secondary antibody diluted in control solution. Cells were collected by centrifugation ($200 \times g$ for 10 min at 4°C), washed twice with PBS and fixed in 0.5 ml of 1% paraformaldehyde in PBS.

Flow Cytometry

All specimens were analyzed in a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). For each sample, 10,000 cells were recorded in list mode and registered in a logarithmic scale. PMN were characterized by forward and side light scattering characteristics, which are indexes of cell size and granularity, respectively, and dot plots were gated for PMN. The mean fluorescence intensity (MFI) and percentage of positive cells in this area was calculated after plotting the FITC fluorescence histograms. Nonspecific fluorescence was any fluorescence associated with PMN incubated with the secondary antibody.

Statistical Analysis

The statistical analysis was based on a mixed model with cow as random effect. Time was split into three periods: 1) before infusion, 2) from infusion until 6 h, and 3) from 8 until 24 h after LPS administration. Data were not statistically evaluated from 24 h onwards. The first analysis addressed the question as to whether there was a linear change (increase or decrease) in function of time during period 2 by adding time as a continuous fixed effect to the mixed model. The second analysis involved testing whether there was an overall difference between period 1 and period 3 by adding periods as categorical fixed effects to the mixed model. Analyses were performed for temperature, cortisol, blood leukocyte count, and SCC, as well as for expression of CD62L,

CD11b, and CD18 (MFI and percentage of positive cells). All these parameters were tested at $\alpha = 0.05$.

RESULTS

Clinical Symptoms

Intramammary infusion with endotoxin in both quarters of the left udder in six early lactating cows resulted in clinical mastitis. Local symptoms such as udder swelling and pain of the infused quarters, accompanied by the appearance of flecks and milk leakage in quarters injected with endotoxin were observed from 3 up to 6 h postinfection. Milk production declined by circa 80% at 24 h postchallenge in the quarters infused with endotoxin. Clinical signs of mastitis disappeared around 72 h postchallenge.

In the second period, rectal temperature increased significantly ($P < 0.001$; Table 1) from a mean of $38.6 \pm 0.06^\circ\text{C}$ at infusion (0 h) to $42.0 \pm 0.16^\circ\text{C}$ at 6 h after endotoxin administration (Figure 1). The pyretic response was detectable from 8 up to 24 h after infusion, as compared with preadministration levels ($P = 0.002$). From then on, fever decreased steadily, reaching physiological values about 24 h postinfusion.

Cortisol Concentration

The temporal changes in cortisol levels from endotoxin-infused cows are shown in Figure 1. The mean plasma cortisol concentration in control samples obtained at the time of inoculation (0 h) was 3.75 ± 0.54 nM. Cortisol levels increased significantly from 0 to 6 h ($P < 0.001$), peaked at 89.6 ± 11.4 nM at 5 h following treatment, and persisted elevated from 8 up to 24 h compared with preinfusion levels ($P < 0.001$). Preadministration values were regained circa 24 h after endotoxin challenge.

Changes in Peripheral Blood Leukocytes and SCC

Total leukocyte count diminished to a significant degree between 0 and 6 h postchallenge ($P < 0.001$). Leukocyte numbers did not differ significantly from 8 up to 24 h after LPS administration compared with preinfusion values. Leukopenia was pronounced, with an abrupt decline to minimal values of circa 74% at 7 h after LPS administration (Figure 2, top). Thenceforth, counts gradually recovered and further increased to about 98% over preinfusion values between 24 and 48 h. Leukocytosis diminished thereafter, though leukocyte numbers remained elevated by the end of the experimental trial compared with prechallenge counts.

Table 1. The effect of endotoxin infusion on temperature, cortisol levels, leukocyte numbers, SCC, and expression of CD62L, CD11b, and CD18 (mean fluorescence intensity and percentage of positive cells) throughout the early inflammatory reaction. Values are means \pm SEM of six cows.

Parameter	First analysis— Linear change from 0 to 6 h		Second analysis— Preinfusion vs. 8 to 24 h		
	Slope	P value	Preinfection	8 to 24 hours	P value
Temperature ($^{\circ}$ C)	0.64 ± 0.04	<0.001	38.6 ± 0.27	39.6 ± 0.19	0.002
Cortisol (nM)	17.4 ± 2.12	<0.001	3.64 ± 5.80	35.7 ± 4.21	<0.001
Leukocyte count (10^6 cells/ml)	-0.96 ± 0.12	<0.001	6.33 ± 0.91	4.82 ± 0.64	0.144
SCC (10^6 cells/ml)	0.70 ± 0.18	<0.001	0.09 ± 0.50	8.32 ± 0.48	<0.001
MFI of CD62L	-1.81 ± 0.47	0.001	37.9 ± 3.45	19.5 ± 3.46	<0.001
CD62L ⁺ (%)	-1.64 ± 0.39	<0.001	98.1 ± 0.80	92.5 ± 0.80	<0.001
MFI of CD11b	3.85 ± 0.39	<0.001	44.6 ± 2.41	64.4 ± 2.42	<0.001
CD11b ⁺ (%)	-0.51 ± 0.23	0.045	99.2 ± 0.49	97.0 ± 0.49	0.001
MFI of CD18	0.54 ± 0.18	0.007	26.4 ± 1.07	26.4 ± 1.08	0.954
CD18 ⁺ (%)	-0.68 ± 0.14	<0.001	99.5 ± 0.44	97.3 ± 0.45	<0.001

The SCC rose steeply up to maximal values 12 h following treatment. Milk cell numbers increased significantly during the first 6 h after infusion ($P < 0.001$). The numbers of cells in milk remained significantly elevated from 8 up to at least 24 h postinfusion ($P < 0.001$) compared with preadministration counts. By the end of the experiment, the SCC was nearly completely recovered (Figure 2, bottom).

Effect of Endotoxin on Blood PMN Numbers and Maturity

PMN counts closely followed total leukocyte counts (Figure 2, top). The number of circulating PMN decreased sharply about 72% at 6 h after endotoxin administration. Thereafter, PMN numbers increased circa 97% over preinfusion levels between 24 and 48 h after LPS challenge. PMN counts remained high at the

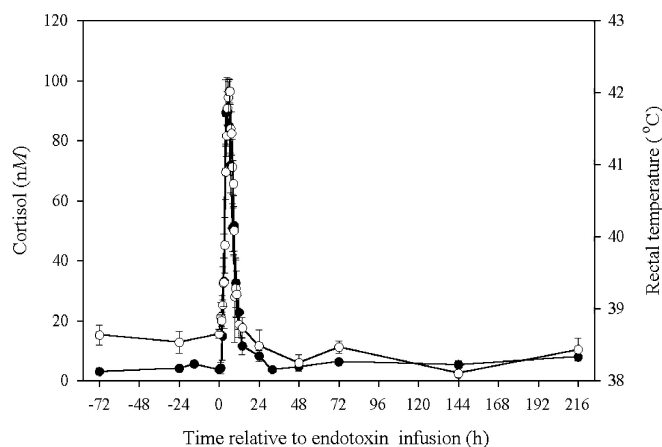


Figure 1. Changes in rectal temperature ($^{\circ}$ C) (○) and blood cortisol levels (nM) (●) in cows intramammarily infused with lipopolysaccharide. Data are means \pm SEM of six cows.

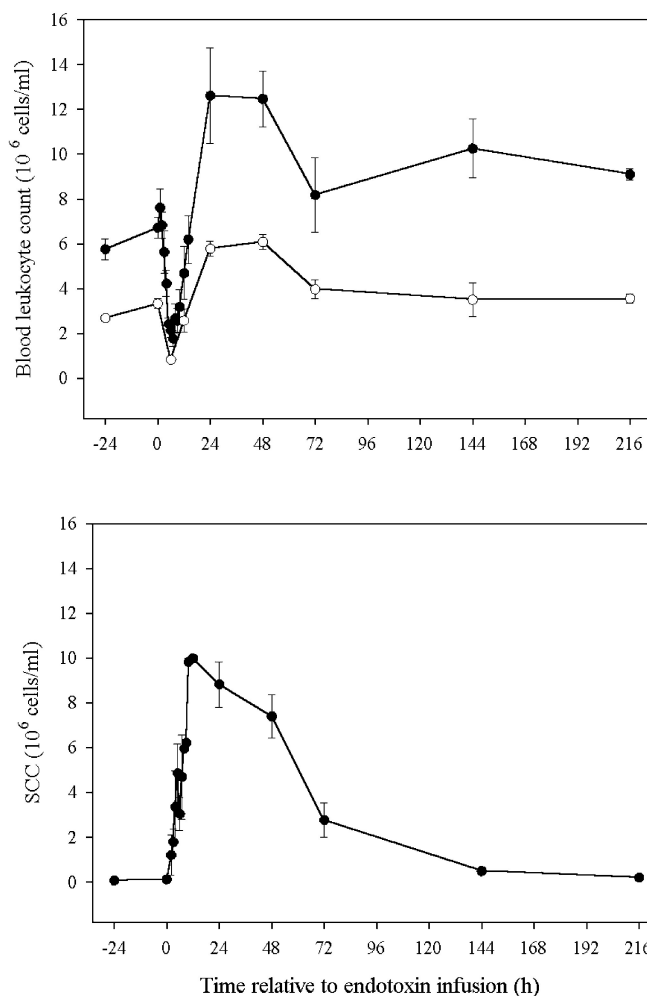


Figure 2. Changes in total leukocyte (●) and polymorphonuclear leukocyte count (○) in blood (top), and SCC in infused quarters (bottom) of cows intramammarily infused with lipopolysaccharide. Data are means \pm SEM of six cows.

Table 2. Changes in the percentage of mature, band, and immature (i.e., myelocytes and metamyelocytes) circulating polymorphonuclear leukocyte (PMN) during lipopolysaccharide administration. Data are means \pm SEM of six cows.

Time (h)	Mature PMN (%)	Band PMN (%)	Immature PMN (%)
-24	46.9 \pm 1.50	0.16 \pm 0.16	0.00 \pm 0.00
0	49.4 \pm 1.04	0.17 \pm 0.11	0.00 \pm 0.00
6	20.9 \pm 2.88	9.53 \pm 1.35	0.76 \pm 0.29
12	7.66 \pm 0.89	24.8 \pm 6.55	10.8 \pm 4.51
24	35.2 \pm 5.06	7.75 \pm 3.50	2.46 \pm 1.21
48	41.4 \pm 2.45	4.37 \pm 2.23	0.65 \pm 0.17
72	48.6 \pm 5.11	2.34 \pm 0.63	0.27 \pm 0.06
144	36.5 \pm 7.48	0.32 \pm 0.15	0.03 \pm 0.03
216	46.9 \pm 1.02	0.11 \pm 0.06	0.04 \pm 0.03

end of the experimental period as compared to preadministration values.

The percentage of mature PMN decreased from circa 49% at the time of infusion to minimal values of about 7.5% at 12 h after endotoxin administration (Table 2). From then on, the percentage of mature PMN gradually recovered to normal values between 48 and 72 h postinfusion. Inversely, percentages of circulating band and immature PMN increased at 6 h and peaked at 12 h postinfusion, diminishing gradually afterwards.

CD62L Expression on Circulating PMN

Following endotoxin administration, the MFI of CD62L on circulating PMN initially decreased (Figure 3, top). From the time of infusion up to 6 h later, a significant decrease in CD62L levels ($P = 0.001$) was observed. Values remained significantly low from 8 until at least 24 h after LPS administration compared with preinfusion labeling of CD62L on PMN ($P < 0.001$). A minimal CD62L density of circa 42% was detected between 10 and 12 h. Thereafter, CD62L MFI increased gradually, regaining preinfusion values between 72 and 144 h after endotoxin administration.

The percentage of CD62L⁺ PMN was circa 95% before endotoxin administration (Figure 3, bottom). A significant decrease between 0 and 6 h postinfusion ($P < 0.001$) was noticed. The percentage of CD62L⁺ PMN remained significantly low from 8 up to 24 h compared with preinfusion values ($P < 0.001$). Minimal values of circa 88% were reached between 6 and 12 h, and control values were regained at approximately 48 h postinfusion.

CD11b and CD18 Expression on Circulating PMN

The MFI of CD11b on circulating PMN increased steadily upon LPS administration (Figure 4, top). A significant increase was detected from 0 up to 6 h postinfusion ($P < 0.001$) compared with values preceding the intramammary challenge. Receptor density remained

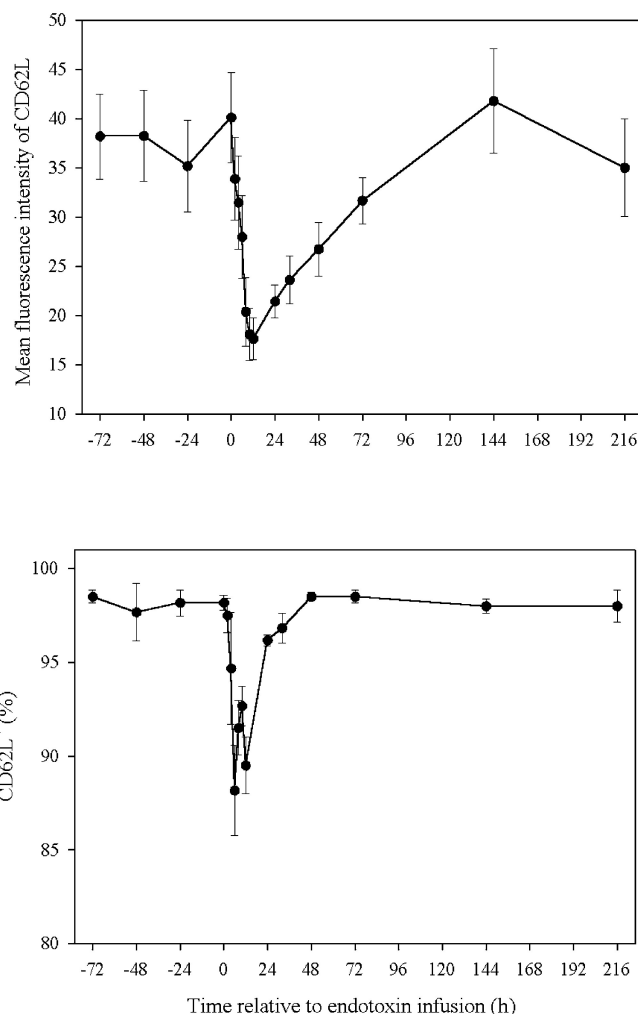


Figure 3. Changes in the mean fluorescence intensity (top) and percentage of positive cells (bottom) of CD62L on circulating polymorphonuclear leukocyte of cows intramammarily infused with lipopolysaccharide. Data are means \pm SEM of six cows.

significantly higher at least from 8 until 24 h after endotoxin infusion compared with preadministration levels ($P < 0.001$). A maximal rise of nearly 56% was observed at 6 h postinfusion. The density of CD11b plateaued between 6 and 12 h, gradually decreasing from then on to prechallenge values at around 48 h after endotoxin administration. Although less pronounced, a significant increase was also detected for the MFI of CD18 between 0 and 6 h postinfusion ($P = 0.007$). Maximal levels were detected at 6 h after LPS administration, with a small increase of circa 10%. The density of CD18 did not significantly differ between 8 and 24 h compared with preinfusion values. Control CD18 MFI was reached earlier compared with CD11b density. A low correlation ($r^2 = -0.06$) existed between the receptor

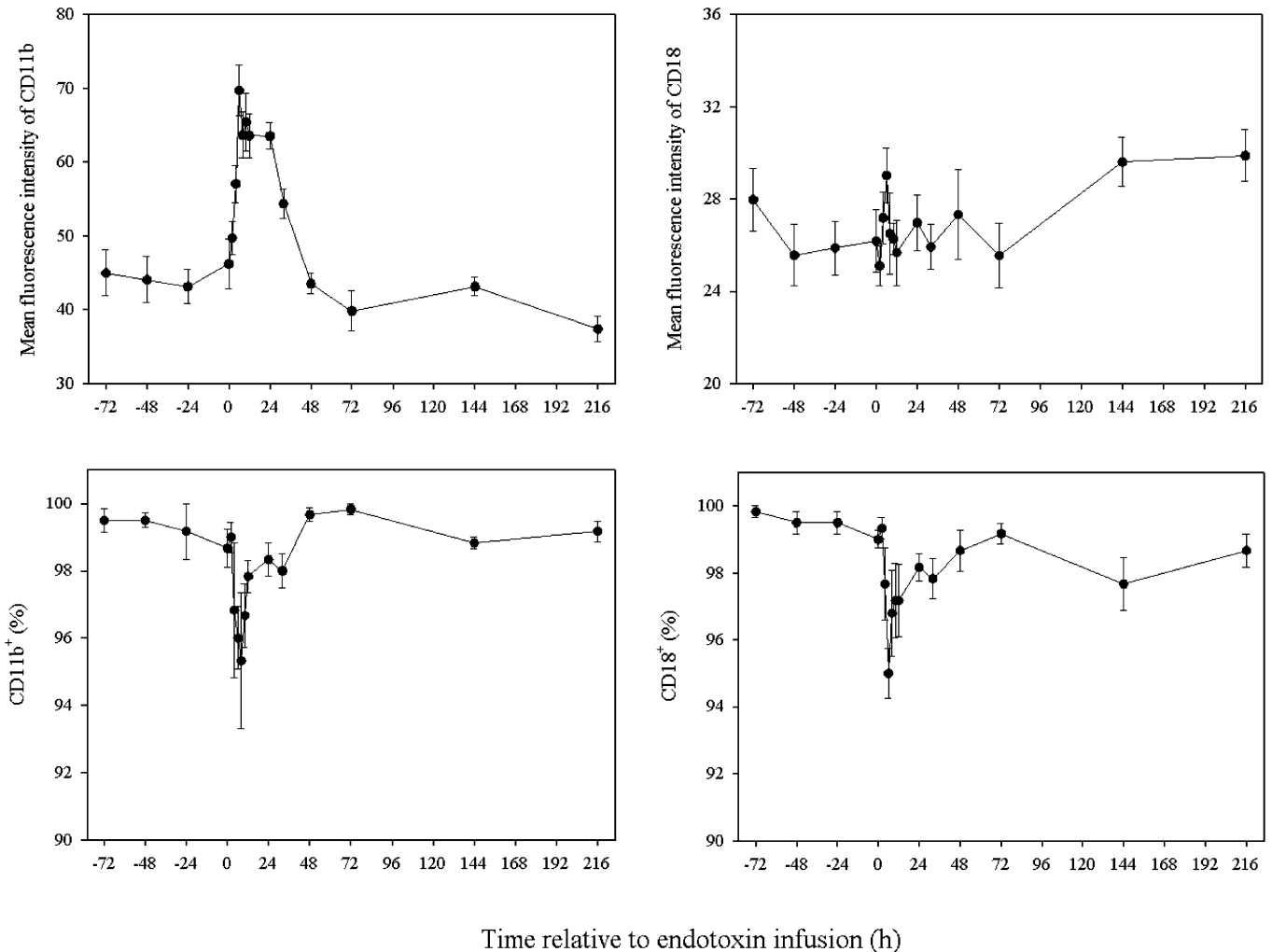


Figure 4. Changes in the mean fluorescence intensity (top) and percentage of positive cells (bottom) of CD11b and CD18 on circulating polymorphonuclear leukocyte of cows intramammarily infused with lipopolysaccharide. Data are means \pm SEM of six cows.

density of CD11b and CD18 during the endotoxin-induced inflammatory response.

The percentage of CD11b⁺ and CD18⁺ PMN was circa 99% for both subunits before challenge (Figure 4, bottom). Positive PMN numbers declined from 0 up to 6 h postinfusion ($P = 0.045$ and $P < 0.001$ for CD11b and CD18, respectively). The percentage of positive cells remained significantly low, at least from 8 up to 24 h after endotoxin administration for both subunits compared with preinfusion values ($P = 0.001$ and $P < 0.001$ for CD11b and CD18, respectively).

DISCUSSION

This study demonstrates that intramammarily infused LPS may modulate the surface expression of the CD62L, CD11b, and CD18 adhesion molecules on blood

PMN. Following endotoxin administration, an inverse regulation of CD62L and the β_2 -integrin subunits was flow cytometrically detected. A rapid decrease of CD62L levels with a concomitant increase of CD11b and CD18 fluorescence intensity was observed at the PMN surface. Our findings closely match those obtained in studies involving clinical gram-negative mastitis (Dosogne et al., 1997; Monfardini et al., 1999; Roets et al., 1999; Diez-Fraile et al., 2000b), as well as LPS mastitis (Yagi et al., 2002). In contrast with the results presented here, Paape et al. (1996) reported that intramammary LPS infusion does not affect CD18 density on bovine circulating PMN. The apparent difference between the outcomes of the two experiments might be caused by the amount of LPS infused.

The endotoxin-caused changes of adhesion molecule density displayed kinetics similar to those observed in

challenges previously performed with *E. coli* (Dosogne et al., 1997; Monfardini et al., 1999; Roets et al., 1999; Diez-Fraile et al., 2000b), except that after LPS infusion an accelerated response of circa 6 h was observed. Indeed, other authors have already pointed out that LPS also elicits a faster response for other parameters altered during gram-negative mastitis (Dosogne, 1998; Hoeben et al., 2000). Because bacteria must first multiply, the delay of adhesion molecule activation might be related to macrophage processing of the live *E. coli*. It has recently been suggested that endogenous soluble CD14 in milk, which binds LPS, might be important in initiating a fast systemic inflammatory response by means of rapidly increasing the release of proinflammatory mediators at the epithelial cell level (Wang et al., 2002). As a matter of fact, LPS is presumably not resorbed from the infected mammary gland into the circulation during endotoxin-induced mastitis (Lohuis et al., 1988). Therefore, major changes in adhesion molecule density observed during the present study are probably related to the release of secondary inflammatory mediators. The chemoattractant tumor necrosis factor (TNF)- α is released into the blood early after LPS infusion (Sordillo and Peel, 1992; Shuster et al., 1993, 1996; Hoeben et al., 2000), and might potentially upregulate CD11b on bovine PMN (Diez-Fraile et al., 2000a). However, the net adhesion molecule variation on the PMN surface is most likely related to a combination of TNF- α with several mediators such as interleukin (IL)-1, IL-6, IL-8, platelet-activating factor, and complement factor C5a, which play a cardinal role in the regulation of PMN activation and recruitment during the acute-phase response (Persson et al., 1993; Shuster et al., 1993, 1995; Ohtsuka et al., 1997; Barber and Yang, 1998; Hoeben et al., 2000).

A slight but significant drop in the percentage of PMN staining positive for CD62L, CD11b, and CD18 was detected following LPS mastitis. This decrease in the percentage of CD62L⁺ PMN was nearly twice as large as the drop in the percentage of CD11b⁺ and CD18⁺ PMN. The results generally agree with those obtained in previous studies performed in cows with mastitis (Paape et al., 1996; Roets et al., 1999; Riollot et al., 2000). In addition, the drop in the CD62L⁺ PMN is consistent with experiments evaluating the effect of cortisol on circulating PMN (Burton et al., 1995). This decrease in positive PMN seems to be related to the appearance of immature cells in blood. Indeed, it has recently been shown that CD11b is expressed in a lower percentage of bone marrow immature PMN compared with the mature pool (Van Merris et al., 2002). This rather small population of PMN staining negative for CD11b and CD18 might not be able to migrate to the inflammatory tissue. In addition, the relevance of this

population in the clearance of the infection would be minimal, because immature bovine PMN have decreased phagocytosis and oxidative burst activity (Van Merris et al., 2002).

Two previous studies at our laboratory showed a high correlation between CD11b and CD18 kinetics during *E. coli* mastitis ($r^2 = 0.78$ and $r^2 = 0.64$, unpublished observations). Indeed, it has been suggested that CD11b is the predominant α subunit of the β_2 -integrins on PMN. Therefore, its expression level has been associated with net variations of the CD18 β subunit (Arnaout, 1990). However, in the present study no interrelationship could be detected between CD11b and CD18 density on PMN after intramammary infusion with LPS. It is possible that a stronger downregulatory effect on CD11a and/or CD11c subunits during endotoxin infusion compared with *E. coli* mastitis could have caused the small net variation in total CD18. As a matter of fact, diminished CD11a expression level on bovine PMN has been previously described during *E. coli* mastitis (Dosogne et al., 1997).

Some of the parameters that are often associated with adhesion molecule mobilization on PMN during endotoxin mastitis are rectal temperature, plasma cortisol concentration, SCC, the number of blood PMN, and the percentage of immature and mature PMN in circulation. Because fairly small variations of CD18 expression levels were observed in the present study, the molecule CD18 was not further considered here. During the initial inflammatory reaction, a sharp decrease in circulating PMN numbers was detected concomitantly with the drop in CD62L and the rise in CD11b density on PMN. The inverse regulation of the adhesion molecule level indicates that the PMN is in an activated state. Indeed, SCC was steadily increasing, which suggested that a fast migratory process of PMN towards the mammary gland had been initiated. The highest rectal temperature was observed 6 h postadministration, a fact that supposedly indicates that the maximal inflammatory response had been reached. As a matter of fact, fever is initiated as a response to the presence of certain proinflammatory cytokines in blood (Kluger, 1991). Moreover, the cortisol level in the blood had peaked already at 5 h postinfusion, likely marking the early onset of the anti-inflammatory process. Indeed, from then on blood PMN numbers were progressively recovering, despite the elevated expression of CD11b. Although increased levels of CD11b on PMN are usually associated with a state of high affinity for endothelial cells, the concomitant downregulation of CD62L might slow down PMN rolling along the endothelium (Luciskas and Gimbrone, 1996; McGill et al., 1996), thereby contributing to the observed increase in circulating PMN counts. The further decrease of CD62L density

to minimal levels and the plateau value reached for CD11b between 6 and 12 h postinfusion is probably related to a combination of activated PMN and the release of cells from the bone marrow immature pool, which peaks 12 h postinfusion. Indeed, it has been reported in humans that immature cells express fewer amounts of the adhesion molecules under study (Lund-Johansen and Terstappen, 1993; Van Eeden et al., 1997). Alternatively, it is equally possible that certain inflammatory mediators released between 6 and 12 h after endotoxin administration may selectively activate CD62L shedding but not CD11b mobilization. This argument is in agreement with experiments in humans and bovines that point to the existence of different activation pathways for the two molecules (Molad et al., 1994; Diez-Fraile et al., 2003). The anti-inflammatory mediator cortisol could potentially have also contributed to the time-lapse of CD62L and CD11b density on circulating PMN, because this phenomenon has also been observed after glucocorticoid administration in peripheral bovine PMN (Burton et al., 1995). From 12 h on, CD62L and CD11b expression returned to control values, a fact which may be explained by the new release of PMN from the mature bone marrow pool together with an insufficient signal to efficiently mobilize CD62L and CD11b. The deactivation mechanism of the β_2 -integrins may be partly mediated by cortisol, which probably initially acts at the peripheral level by inhibiting further upregulation of CD11/CD18 adhesion molecules by proinflammatory stimuli (Filep et al., 1997; Roets et al., 1999; Diez-Fraile et al., 2000a), and later at the bone marrow compartment by downregulating the transcriptional induction (Burton et al., 1995; Burton and Kehrli, 1995).

CONCLUSION

In conclusion, receptor density of CD62L, CD11b, and CD18 on blood PMN is affected by intramammary LPS infusion most probably related to the induction of secondary proinflammatory mediators, and undergoes an accelerated response compared with previously performed experiments during coliform mastitis. Moreover, differences between endotoxin- and *E. coli*-induced mastitis on adhesion molecule expression levels may arise from the fact that CD11b and CD18 density were not correlated during endotoxin mastitis. This study contributes to a better understanding of adhesion molecule kinetics on bovine PMN during the inflammatory response initiated by intramammarily administered endotoxins.

ACKNOWLEDGMENTS

The authors express their appreciation to E. Vander Elstraeten and L. De Bruyne for their technical assis-

tance. They also thank M. J. Paape (Immunology and Disease Resistance Laboratory, USDA-ARS, Beltsville, MD) for his generous gift of the 11G10 mAb. This study was funded by a Marie Curie Research Training Grant from the European Community (no. FAIR-BM-974214) and the Exploring European Research grant from the Ghent University (no. 011V0401).

REFERENCES

- Arnaout, M. A. 1990. Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood* 75:1037–1050.
- Barber, M. R., and T. J. Yang. 1998. Chemotactic activities in nonmastitic and mastitic mammary secretions: Presence of interleukin-8 in mastitic but not nonmastitic secretions. *Clin. Diagn. Lab. Immunol.* 5:82–86.
- Burton, J. L., and M. E. Kehrli, Jr. 1995. Regulation of neutrophil adhesion molecules and shedding of *Staphylococcus aureus* in milk of cortisol- and dexamethasone-treated cows. *Am. J. Vet. Res.* 56: 997–1006.
- Burton, J. L., M. E. Kehrli Jr, S. Kapil, and R. L. Horst. 1995. Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: Effects of cortisol and dexamethasone. *J. Leukoc. Biol.* 57: 317–325.
- Diez-Fraile, A., E. Meyer, A. M. Massart-Leën, and C. Burvenich. 2000a. Effect of isoproterenol and dexamethasone on the lipopolysaccharide induced expression of CD11b on bovine neutrophils. *Vet. Immunol. Immunopathol.* 76:151–156.
- Diez-Fraile, A., G. M. Tomita, E. Meyer, M. J. Paape, and C. Burvenich. 2000b. CD62L expression on circulating neutrophils in relation to *Escherichia coli* mastitis. Pages 177–178 in *Proc. IDF Symp. Immunol. Ruminant Mammary Gland*, Stresa, Italy.
- Diez-Fraile, A., E. Meyer, and C. Burvenich. 2002. Regulation of adhesion molecules on circulating neutrophils during coliform mastitis and their possible immunomodulation with drugs. *Vet. Immunol. Immunopathol.* 86:1–10.
- Diez-Fraile, A., E. Meyer, M. J. Paape, and C. Burvenich. 2003. Analysis of selective mobilization of L-selectin and Mac-1 reservoirs in bovine neutrophils and eosinophils. *Vet. Res.* 34:57–70.
- Dosogne, H., C. Burvenich, T. Van Werven, E. Roets, E. N. Noordhuizen-Stassen, and B. Goddeeris. 1997. Increased surface expression of CD11b receptors on polymorphonuclear leukocytes is not sufficient to sustain phagocytosis during *Escherichia coli* mastitis in early postpartum dairy cows. *Vet. Immunol. Immunopathol.* 60:47–59.
- Dosogne, H. 1998. Defense against intramammary *Escherichia coli* infections by bovine neutrophil leukocytes during the early postpartum period: Phagocytosis and detoxification of endotoxins. Ph.D. Diss., Ghent Univ., Belgium.
- Filep, J. G., A. Delalandre, Y. Payette, and E. Földes-Filep. 1997. Glucocorticoid receptor regulates expression of L-selectin and CD11/CD18 on human neutrophils. *Circulation* 96:295–301.
- Hill, A. W. 1994. *Escherichia coli* mastitis. Pages 117–133 in *Escherichia coli* in Domestic Animals and Humans. 1st ed. C. L. Gyles, ed. CAB International, Wallingford, UK.
- Hoeben, D., C. Burvenich, E. Trevisi, G. Bertoni, J. Hamann, R. M. Bruckmaier, and J. W. Blum. 2000. Role of endotoxin and TNF- α in the pathogenesis of experimentally induced coliform mastitis in periparturient cows. *J. Dairy Res.* 67:503–514.
- Kluger, M. J. 1991. Fever: role of pyrogens and cryogens. *Physiol. Rev.* 71:93–127.
- Letesson, J. J., and M. Delcommenne. 1993. Production of a monoclonal antibody to the light chain of the bovine β_2 -integrin family (BoCD18). *Vet. Immunol. Immunopathol.* 39:103–108.
- Lohuis, J. A. C. M., J. H. M. Verheijden, C. Burvenich, and A. S. J. P. A. M. Van Miert. 1988. Pathophysiological effects of endotoxins in ruminants. 1. Changes in body temperature and reticulo-rumen motility, and the effect of repeated administration. *Vet. Q.* 10:109–116.

- Luscinskas, F. W., and M. A. Gimbrone, Jr. 1996. Endothelial-dependent mechanisms in chronic inflammatory leukocyte recruitment. *Annu. Rev. Med.* 47:413–421.
- Lund-Johansen, F., and L. W. M. M. Terstappen. 1993. Differential surface expression of cell adhesion molecules during granulocyte maturation. *J. Leukoc. Biol.* 54:47–55.
- McGill, S. N., N. A. Ahmed, F. Hu, R. P. Michel, and N. V. Christou. 1996. Shedding of L-selectin as a mechanism for reduced polymorphonuclear neutrophil exudation in patients with the systemic inflammatory response syndrome. *Arch. Surg.* 131:1141–1147.
- Molad, Y., K. A. Haines, D. C. Anderson, J. P. Buyon, and B. N. Cronstein. 1994. Immunocomplexes stimulate different signaling events to chemoattractants in the neutrophil and regulate L-selectin and β_2 -integrin expression differently. *Biochem. J.* 299:881–887.
- Monfardini, E., C. Burvenich, A. M. Massart-Leën, E. Smits, and M. J. Paape. 1999. Effect of antibiotic induced bacterial clearance in the udder on L-selectin shedding of blood neutrophils in cows with *Escherichia coli* mastitis. *Vet. Immunol. Immunopathol.* 67:373–384.
- Ohtsuka, H., K. Ohki, T. Tanaka, M. Tajima, T. Yoshino, and K. Takahashi. 1997. Circulating tumor necrosis factor and interleukin-1 after administration of LPS in adult cows. *J. Vet. Med. Sci.* 59:927–929.
- Paape, M. J., E. M. Lilius, P. A. Wiitanen, M. P. Kontio, and R. H. Miller. 1996. Intramammary defense against infections induced by *Escherichia coli* in cows. *Am. J. Vet. Res.* 57:477–482.
- Persson, K., I. Larsson, and C. Hallen Sandgren. 1993. Effects of certain inflammatory mediators on bovine neutrophil migration in vivo and in vitro. *Vet. Immunol. Immunopathol.* 37:99–112.
- Riollet, C., P. Rainard, and B. Poutrel. 2000. Kinetics of cells and cytokines during immune-mediated inflammation in the mammary gland of cows systemically immunized with *Staphylococcus aureus* α -toxin. *Inflamm. Res.* 49:486–496.
- Roets, E., C. Burvenich, A. Diez-Fraile, and E. N. Noordhuizen-Stassen. 1999. Evaluation of the role of endotoxin and cortisol on modulation of CD18 adhesion receptors in cows with mastitis caused by *Escherichia coli*. *Am. J. Vet. Res.* 60:534–540.
- Shuster, D. E., E. K. Lee, and M. E. Kehrli Jr. 1996. Bacterial growth, inflammatory cytokine production, and neutrophil recruitment during coliform mastitis in cows within ten days after calving, compared with cows at midlactation. *Am. J. Vet. Res.* 57:1569–1575.
- Shuster, D. E., and M. E. Kehrli, Jr. 1995. Administration of recombinant human interleukin 1 receptor antagonist during endotoxin-induced mastitis in cows. *Am. J. Vet. Res.* 56:313–320.
- Shuster, D. E., M. E. Kehrli, Jr., and M. G. Stevens. 1993. Cytokine production during endotoxin-induced mastitis in lactating dairy cows. *Am. J. Vet. Res.* 54:80–85.
- Smits, E., C. Burvenich, A. J. Guidry, and A. M. Massart-Leën. 2000. Adhesion receptor CD11b/CD18 contributes to neutrophil diapedesis across the bovine blood-milk barrier. *Vet. Immunol. Immunopathol.* 73:255–265.
- Sordillo, L. M., and J. E. Peel. 1992. Effect of interferon- γ on the production of tumor necrosis factor during acute *Escherichia coli* mastitis. *J. Dairy Sci.* 75:2119–2125.
- Sulón, J., L. Demey-Ponsart, P. Beauduin, and J. C. Sodoyez. 1978. Radioimmunoassay of corticosterone, cortisol and cortisone: Their application to human cord and maternal plasma. *J. Steroid. Biochem.* 9:671–676.
- Van Eeden, S. F., R. Miyagashima, L. Haley, and J. C. Hogg. 1997. A possible role for L-selectin in the release of polymorphonuclear leukocytes from bone marrow. *Am. J. Physiol.* 272:H1717–1724.
- Van Merris, V., E. Meyer, and C. Burvenich. 2002. Functional maturation during bovine granulopoiesis. *J. Dairy Sci.* 85:2859–2868.
- Wang, Y., M. J. Paape, L. Leino, A. V. Capuco, and H. Närvä. 1997. Functional and phenotypic characterization of monoclonal antibodies to bovine L-selectin. *Am. J. Vet. Res.* 58:1392–1401.
- Wang, Y., D. S. Zarlenga, M. J. Paape, and G. E. Dahl. 2002. Recombinant bovine soluble CD14 sensitizes the mammary gland to lipopolysaccharide. *Vet. Immunol. Immunopathol.* 86:115–124.
- Yagi, Y., H. Shiono, T. Shibahara, Y. Chikayama, I. Nakamura, and A. Ohnuma. 2002. Increase in apoptotic polymorphonuclear neutrophils in peripheral blood after intramammary infusion of *Escherichia coli* lipopolysaccharide. *Vet. Immunol. Immunopathol.* 89:115–125.